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INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

INTEGRIN ALPHA IIB BETA 3 SPECIFIC ANTIBODIES AND PEPTIDES

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☐ Application Date Sheet. See 37 CFR 1.76

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Respectfully submitted,

SIGNATURE Thomas E Northrup

Date December 3, 2003

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Applicant: Barbas, et al.

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Title: INTEGRIN $\alpha_{IIB}\beta_3$ SPECIFIC ANTIBODIES AND PEPTIDES

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INTEGRIN $\alpha_{IIb}\beta_3$ SPECIFIC ANTIBODIES AND PEPTIDES

Technical Field of the Invention

The field of this invention is platelet aggregation. More particularly, the present invention pertains to integrin specific antibodies and peptides and the use of those compounds for inhibiting platelet aggregation.

Background of the Invention

Integrin $\alpha_{IIb}\beta_3$ inhibitors are new class of antithrombotic agents that block fibrinogen binding to the platelet integrin $\alpha_{IIb}\beta_3$, thereby inhibiting platelet-platelet interactions essential for the formation of platelet thrombi (Topol, E. J., Byzova, T. V., and Plow, E. F. (1999) Platelet GPIIb-IIIa blockers. *Lancet* 353, 227-231; Coller, B. S. (1997) Platelet GPIIb/IIIa antagonists: the first anti-integrin receptor therapeutics. *J Clin Invest* 99, 1467-1471). Integrin $\alpha_{IIb}\beta_3$ inhibitors are used for the management of patients with non-ST-segment elevation acute coronary syndromes and patients undergoing percutaneous coronary intervention (Proimos, G. (2001) Platelet aggregation inhibition with glycoprotein IIb-IIIa inhibitors. *J Thromb Thrombolysis* 11, 99-110). Among these inhibitors, abciximab (ReoPro, Centocor, Inc., Malvern, Pennsylvania, and Eli Lilly & Company, Indianapolis, Indiana) (Coller, B. S. (1997) Platelet GPIIb/IIIa antagonists: the first anti-integrin receptor therapeutics. *J Clin Invest* 99, 1467-1471), eptifibatide (INTEGRILIN, COR Therapeutics, Inc., South San Francisco, California, and Key Pharmaceuticals, Inc., Kenilworth, New Jersey) (Phillips, D. R., and Scarborough, R. M. (1997) Clinical pharmacology of eptifibatide. *Am J Cardiol* 80, 11B-20B; Scarborough, R. M. (1999) Development of eptifibatide. *Am Heart J* 138, 1093-1104), and tirofiban (Aggrastat, Merck & Co., Inc., Whitehouse Station, New Jersey) (Vickers, S., Theoharides, A. D., Arison, B., Balani, S. K., Cui, D., Duncan, C. A., Ellis, J. D., Gorham, L. M., Polsky, S. L., Prueksaritanont, T., Ramjit, H. G., Slaughter, D.

E., and Vyas, K. P. (1999) In vitro and in vivo studies on the metabolism of tirofiban. *Drug Metab Dispos* 27, 1360-1366) are clinically approved in the United States. Abciximab, the first approved and most widely used agent, is a chimeric Fab with mouse variable and human constant domains. It binds to an epitope adjacent to the ligand binding region and inhibits fibrinogen binding by steric hindrance. Abciximab was reported to cross-react with integrin $\alpha_v\beta_3$ and $\alpha_M\beta_2$ (Scarborough, R. M. (1999) Development of eptifibatide. *Am Heart J* 138, 1093-1104). Eptitibatide and tirofiban, on the other hand, are small molecule drugs that bind to the RGD ligand interaction site of the integrin and are $\alpha_{IIb}\beta_3$-specific. They showed lower affinity and much shorter circulatory half-lives than abciximab (Scarborough, R. M. (1999) Development of eptifibatide. *Am Heart J* 138, 1093-1104; Vickers, S., Theoharides, A. D., Arison, B., Balani, S. K., Cui, D., Duncan, C. A., Ellis, J. D., Gorham, L. M., Polsky, S. L., Prueksaritanont, T., Ramjit, H. G, Slaughter, D. E., and Vyas, K. P. (1999) In vitro and in vivo studies on the metabolism of tirofiban. *Drug Metab Dispos* 27, 1360-1366; McClellan, K. J., and Goa, K. L. (1998) Tirofiban. A review of its use in acute coronary syndromes. *Drugs* 56, 1067-1080).

Acute thrombocytopenia is a recognized complication of treatment with integrin $\alpha_{IIb}\beta_3$ inhibitors. Thrombocytopenia, often severe (platelets less than 50 X 10^9/L), occurs in 0.5% to 1% of patients given abciximab for the first time (Berkowitz, S. D., Sane, D. C., Sigmon, K. N., Shavender, J. H., Harrington, R. A., Tcheng, J. E., Topol, E. J., and Califf, R. M. (1998) Occurrence and clinical significance of thrombocytopenia in a population undergoing high-risk percutaneous coronary revascularization. Evaluation of c7E3 for the Prevention of Ischemic Complications (EPIC) Study Group. *J Am Coll Cardiol* 32, 311-319; Jubelirer, S. J., Koenig, B. A., and Bates, M. C. (1999) Acute profound thrombocytopenia following C7E3 Fab (Abciximab) therapy: case reports, review of the literature and implications for therapy. *Am J Hematol* 61, 205-208; Kereiakes, D. J., Berkowitz, S. D., Lincoff, A. M., Tcheng, J. E., Wolski, K., Achenbach, R., Melsheimer, R., Anderson, K.,

Califf, R. M., and Topol, E. J. (2000) Clinical correlates and course of thrombocytopenia during percutaneous coronary intervention in the era of abciximab platelet glycoprotein IIb/IIIa blockade. *Am Heart J* 140, 74-80) and in 4% of patients after the second administration (Madan, M., Kereiakes, D. J., Hermiller, J. B., Rund, M. M., Tudor, G., Anderson, L., McDonald, M. B., Berkowitz, S. D., Sketch, M. H., Jr., Phillips, H. R., 3rd, and Tcheng, J. E. (2000) Efficacy of abciximab readministration in coronary intervention. *Am J Cardiol* 85, 435-440; Tcheng, J. E., Kereiakes, D. J., Braden, G. A., Jordan, R. E., Mascelli, M. A., Langrall, M. A., and Effron, M. B. (1999) Readministration of abciximab: interim report of the ReoPro readministration registry. *Am Heart J* 138, S33-38). In clinical trials of tirofiban the incidence ranged from 0.1% to 0.5%, which is only twice the incidence seen in patients not given the drug (The RESTORE Investigators. Randomized Efficacy Study of Tirofiban for Outcomes and REstenosis(1997) Effects of platelet glycoprotein IIb/IIIa blockade with tirofiban on adverse cardiac events in patients with unstable angina or acute myocardial infarction undergoing coronary angioplasty. *Circulation* 96, 1445-1453; Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) Study Investigators. (1998) A comparison of aspirin plus tirofiban with aspirin plus heparin for unstable angina. *N Engl J Med* 338, 1498-1505; Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited by Unstable Signs and Symptoms (PRISM-PLUS) Study Investigators. (1998) Inhibition of the platelet glycoprotein IIb/IIIa receptor with tirofiban in unstable angina and non-Q-wave myocardial infarction. *N Engl J Med* 338, 1488-1497). In the PURSUIT trial of eptifibatide, the incidence was approximately the same in patients given the study drug as in patients given placebo (McClure, M. W., Berkowitz, S. D., Sparapani, R., Tuttle, R., Kleiman, N. S., Berdan, L. G., Lincoff, A. M., Deckers, J., Diaz, R., Karsch, K. R., Gretler, D., Kitt, M., Simoons, M., Topol, E. J., Califf, R. M., and Harrington, R. A. (1999) Clinical significance of thrombocytopenia during a non-ST-elevation acute coronary syndrome. The platelet glycoprotein IIb/IIIa in unstable angina: receptor suppression using

integrilin therapy (PURSUIT) trial experience. *Circulation* 99, 2892-2900). Only a small subset of patients given eptifibatide had profound, unexplained thrombocytopenia (McClure, M. W., Berkowitz, S. D., Sparapani, R., Tuttle, R., Kleiman, N. S., Berdan, L. G., Lincoff, A. M., Deckers, J., Diaz, R., Karsch, K. R., Gretler, D., Kitt, M., Simoons, M., Topol, E. J., Califf, R. M., and Harrington, R. A. (1999) Clinical significance of thrombocytopenia during a non-ST-elevation acute coronary syndrome. The platelet glycoprotein IIb/IIIa in unstable angina: receptor suppression using integrilin therapy (PURSUIT) trial experience. *Circulation* 99, 2892-2900). The cause of thrombocytopenia induced by integrin $\alpha_{IIb}\beta_3$ inhibitors is not known, although recently it was reported that human antibodies against the mouse variable domains of abciximab, presumably induced by the first exposure of abciximab, were the cause of platelet destruction in patients who developed severe thrombocytopenia after being given abciximab a second time (Curtis, B. R., Swyers, J., Divgi, A., McFarland, J. G., and Aster, R. H. (2002) Thrombocytopenia after second exposure to abciximab is caused by antibodies that recognize abciximab-coated platelets. *Blood* 99, 2054-2059).

Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$, and $\alpha_5\beta_1$ bind to a variety of adhesive proteins containing an RGD tripeptide. Exploiting this feature, adhesive protein-mimicking synthetic human mAbs containing an RGD motif in HCDR3 flanked by six randomized residues were selected from antibody libraries by phage display (Barbas, C. F., 3rd, Languino, L. R., and Smith, J. W. (1993) High-affinity self-reactive human antibodies by design and selection: targeting the integrin ligand binding site. *Proc Natl Acad Sci U S A* 90, 10003-10007). Fab-9, the most potent of these antibodies, binds to integrin $\alpha_v\beta_3$ nearly 1,000-fold better than to integrins $\alpha_v\beta_5$ and $\alpha_5\beta_1$. However, neither of the selected antibodies including Fab-9 distinguished between the two β_3 integrins, $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$. Therefore, a motif optimization library was generated to determine whether further rounds of engineering and selection on the RGD motif within the HCDR3 of Fab-9 could produce an antibody with specificity for either

integrin $\alpha_v\beta_3$ or $\alpha_{IIb}\beta_3$ (Smith, J. W., Hu, D., Satterthwait, A., Pinz-Sweeney, S., and Barbas, C. F., 3rd (1994) Building synthetic antibodies as adhesive ligands for integrins. *J Biol Chem* 269, 32788-32795). While many of the selected antibodies showed some preference to either $\alpha_v\beta_3$ or $\alpha_{IIb}\beta_3$, all antibodies still bound to both integrins. The majority of the selected antibodies had the consensus sequence (K/R)XD. Interestingly, the middle position in the RGD motif proved to be highly permissive. It was found that the Gly can be substituted by Val, Ala, Asn, Arg, Thr, Gln, Asp, Ser, and Trp (Smith, J. W., Hu, D., Satterthwait, A., Pinz-Sweeney, S., and Barbas, C. F., 3rd (1994) Building synthetic antibodies as adhesive ligands for integrins. *J Biol Chem* 269, 32788-32795).

The present invention discloses integrin $\alpha_{IIb}\beta_3$ specific human mAbs from a newly designed synthetic human antibody library. First, the two cysteine residues present in the previous libraries were removed from HCDR3. The reduction in HCDR3 length along with the removal of the disulfide bridge potentially allows for more structural flexibility in the HCDR3 loop. The removal of the disulfide bridge was encouraged by the observation that a peptide derived from the HCDR3 of a previously selected antibody (CSFGRGDIRNC) (SEQ ID NO:1) and its linear version GSFGRGDIRNG (SEQ ID NO:2) had nearly identical efficacy in ligand binding assays (Smith, J. W., Hu, D., Satterthwait, A., Pinz-Sweeney, S., and Barbas, C. F., 3rd (1994) Building synthetic antibodies as adhesive ligands for integrins. *J Biol Chem* 269, 32788-32795). Second, we chose RAD rather than RGD as the integrin-binding core motif and randomized six residues in the flanking region. An RAD-containing peptide, GRADSP, has been widely used as inactive control peptide in experiments where RGD-containing peptides are tested (Wu, M. H., Ustinova, E., and Granger, H. J. (2001) Integrin binding to fibronectin and vitronectin maintains the barrier function of isolated porcine coronary venules. *J Physiol* 532, 785-791; Slepian, M. J., Massia, S. P., Dehdashti, B., Fritz, A., and Whitesell, L. (1998) Beta3-integrins rather than beta1-integrins dominate integrin-matrix interactions involved in postinjury smooth muscle cell migration. *Circulation*

97, 1818-1827; Guilherme, A., Torres, K., and Czech, M. P. (1998) Cross-talk between insulin receptor and integrin alpha5 beta1 signaling pathways. *J Biol Chem* 273, 22899-22903; Hautanen, A., Gailit, J., Mann, D. M., and Ruoslahti, E. (1989) Effects of modifications of the RGD sequence and its context on recognition by the fibronectin receptor. *J Biol Chem* 264, 1437-1442; Adderley, S. R., and Fitzgerald, D. J. (2000) Glycoprotein IIb/IIIa antagonists induce apoptosis in rat cardiomyocytes by caspase-3 activation. *J Biol Chem* 275, 5760-5766). However, the fact that we selected RAD from our motif optimization library (Smith, J. W., Hu, D., Satterthwait, A., Pinz-Sweeney, S., and Barbas, C. F., 3rd (1994) Building synthetic antibodies as adhesive ligands for integrins. *J Biol Chem* 269, 32788-32795), suggests that the flanking residues determine whether or whether not RAD motifs can bind to integrins. We hypothesized that the RAD motif embedded in a randomized flanking region might increase the probability of selecting antibodies specific for integrin $\alpha_{IIb}\beta_3$.

Brief Summary of the Invention

Here we used phage display to select monoclonal antibodies specific to integrin $\alpha_{IIb}\beta_3$ from a synthetic human antibody library based on the randomized HCDR3 sequence VGXXXRADXXXYAMDV (SEQ ID NO:3). The selected antibodies revealed a strong consensus in HCDR3 (V(V/W)CRAD(K/R)RC) (SEQ ID NO:4) and high specificity toward integrin $\alpha_{IIb}\beta_3$ but not to other RGD binding integrins like, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$. The selected antibodies as well as three synthetic peptides VWCRADRRC (SEQ ID NO:5), VWCRADKRC (SEQ ID NO:6), and VVCRADRRC (SEQ ID NO:7) whose sequences were derived from the HCDR3 sequences of the selected antibodies strongly inhibited the interaction between integrin $\alpha_{IIb}\beta_3$ and fibrinogen and platelet aggregation *ex vivo*. To our

knowledge these are the first fully human monoclonal antibodies that are specific to integrin $\alpha_{IIb}\beta_3$ and can potently inhibit platelet aggregation.

In one aspect, the present invention provides an isolated and purified peptide inhibitor of platelet aggregation. The peptide has from 9 to about 50 amino acid residues and an amino acid residue sequence that corresponds to V(V/W)CRAD(K/R)RC (SEQ ID NO:4). The peptide preferably includes the amino acid residue sequence of any of SEQ ID NOs: 5-7 and, more preferably, has the amino acid residue sequence of any of SEQ ID NOs: 5-7.

In another aspect, the invention provides an antibody that specifically immunoreacts with $\alpha_{IIb}\beta_3$. The antibody includes the amino acid residue sequence V(R/G)V(V/W)CRAD (R/K)RCYAMDV (SEQ ID NO:8) within a complementarity determining region of the antibody. Preferably, the complementarity determining region is located in a heavy chain of the antibody and, more particularly, the complementarity determining region is HCDR3. The antibody is most preferably a human antibody. Exemplary and preferred antibodies are designated herein as RAD3, RAD4, RAD9, RAD11, RAD12, RAD32, RAD34, RAD87, or RAD88. The invention also contemplates an antibody having the immunoreactivity of any of RAD3, RAD4, RAD9, RAD11, RAD12, RAD32, RAD34, RAD87, or RAD88.

In yet another aspect, this invention provides a method of inhibiting platelet aggregation including the step of contacting platelets with an effective inhibitory amount of a peptide or antibody of the invention.

In still yet another aspect, this invention provides a method of inhibiting binding of fibrinogen to platelets including the step of contacting platelets with an effective inhibitory amount of a peptide or antibody of the invention.

Brief Description of the Drawings

In the drawings that form a portion of the specification

Fig. 1 shows that selected Fabs bind to human integrin $\alpha_{IIb}\beta_3$ but not to other RGD-binding human integrins. Shown are ELISA results based on immobilized human integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ and supernatant containing soluble Fabs expressed by pComb3X-transfected *E. coli*. A rat anti-HA mAb conjugated to HRP was used for detection.

Fig. 2 shows that Fab RAD87 binds to human platelets but not to HUVEC cells. Analysis by flow cytometry showed that Fab RAD87 bound only to human platelets, which express both integrin $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$, but not to HUVEC cells, which mainly express integrin $\alpha_v\beta_3$. By contrast, Fab abciximab bound to both platelets and HUVEC cells, confirming its documented cross-reactivity with the two β_3 integrins. The *y* axis gives the number of events in linear scale, the *x* axis the fluorescence intensity in logarithmic scale.

Fig. 3 shows that Fab RAD87 and synthetic peptides derived from the selected HCDR3 sequences inhibit the interaction between integrin $\alpha_{IIb}\beta_3$ and fibrinogen. Biotinylated fibrinogen was mixed with (A) various concentrations of Fabs RAD87 and abciximab as well as pooled human IgG as negative control and (B) synthetic peptides and added to integrin $\alpha_{IIb}\beta_3$ immobilized on an ELISA plate. Streptavidin-HRP was used for detection. Mean values and standard deviations of three independent experiments are shown.

Fig. 4 shows that Fab RAD87 inhibits platelet aggregation *ex vivo*. The shown platelet aggregation assays were derived from a whole blood lumi-aggregometer. For each assay, 15 µl of Fab RAD87 (A) or Fab abciximab (B) solution was mixed with 435 µl of platelet-rich plasma to reach a final concentration between 20 nM and 100 nM. Then ADP was added to a final concentration of 20 µM and the aggregation was monitored for 10 min. The plotted mean values and standard deviations of three independent experiments are shown in (C).

Fig. 5 shows that synthetic peptides derived from the selected HCDR3 sequences inhibit platelet aggregation *ex vivo*. Platelet aggregation assays were performed as

described in Fig. 4 in the presence of the indicated peptide concentrations. Note that all three cyclic peptides with the RAD motif potently inhibited platelet aggregation (A-C) whereas a linear peptide with the RAD motif and two control peptides with an inversed RAD motif have no effect over background (D). Abciximab Fab was used as positive control in all platelet aggregation assays.

Fig. 6 shows the amino acid residue sequences of the heavy chain of the antibodies of the present invention.

Detailed Description of the Invention

The present invention provides antibodies that immunoreact with integrin $\alpha_{IIb}\beta_3$. The term antibody in its various grammatical forms is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and portions of an immunoglobulin molecule, including those portions known in the art as Fab, Fab', $F(ab')_2$ and F(v).

An antibody of the present invention is made and identified using phage display technology as is well known in the art (See, e.g., International Patent Application Publication WO 94/18221). A detailed description of such procedures is set forth below in the Examples. A preferred antibody is a human antibody.

The present invention describes, in one embodiment, human monoclonal antibodies that contain a binding site as described herein and which bind specifically to a preselected target molecule. The invention also describes cell lines which produce the antibodies, methods for producing the cell lines, and methods for producing the human monoclonal antibodies.

Insofar as a display protein of this invention on a phagemid particle is, in preferred embodiments, a fusion protein between an immunoglobulin heavy or light chain and a filamentous phage membrane anchor, it is to be understood that the display protein is, in effect, an engineered immunoglobulin heavy or light chain into which a binding site has been introduced. Furthermore, in many embodiments, the expression of the display protein is prepared on the phagemid surface as a heterodimer formed between immunoglobulin heavy and light chain peptides, with one or the other being a fusion protein with the membrane anchor. Thus, where the heavy chain is used as the fusion protein, a display protein in preferred embodiments comprises a Fab fragment having an anchored heavy chain associated with a light chain.

The preparation of cell lines producing monoclonal antibodies of the invention is described in great detail further herein, and can be accomplished using the phagemid vector mutagenesis methods described herein, and using routine screening techniques which permit determination of the elementary binding patterns of the monoclonal antibody of interest indicative that the binding site has been produced. Thus, if a human monoclonal antibody being tested binds to the preselected target molecule, then the human monoclonal antibody being tested and the human monoclonal antibody produced by the cell lines of the invention are considered equivalent.

It is also possible to determine, without undue experimentation, if a human monoclonal antibody has the same (i.e., equivalent) specificity as a human monoclonal antibody of this invention by ascertaining whether the former prevents the latter from binding

to a preselected target molecule. If the human monoclonal antibody being tested competes with the human monoclonal antibody of the invention, as shown by a decrease in binding by the human monoclonal antibody of the invention in standard competition assays for binding to the target molecule when present in the solid phase, then it is likely that the two monoclonal antibodies bind to the same, or a closely related, epitope.

Still another way to determine whether a human monoclonal antibody has the specificity of a human monoclonal antibody of the invention is to pre-incubate the human monoclonal antibody of the invention with the target molecule with which it is normally reactive, and then add the human monoclonal antibody being tested to determine if the human monoclonal antibody being tested is inhibited in its ability to bind the target molecule. If the human monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or functionally equivalent, epitopic specificity as the monoclonal antibody of the invention.

An additional way to determine whether a human monoclonal antibody has the specificity of a human monoclonal antibody of the invention is to determine the amino acid residue sequence of the CDR regions of the antibodies in question. Antibody molecules having identical, or functionally equivalent, amino acid residue sequences in their CDR regions have the same binding specificity. Methods for sequencing peptides are well known in the art.

The immunospecificity of an antibody, its target molecule binding capacity, and the attendant affinity the antibody exhibits for the epitope, are defined by the epitope with which the antibody immunoreacts. The epitope specificity is defined at least in part by the amino acid residue sequence of the variable region of the heavy chain of the immunoglobulin the antibody, and in part by the light chain variable region amino acid residue sequence.

Particularly preferred is a human monoclonal antibody having the binding specificity of the monoclonal antibody produced by an E. coli microorganism or produced by a plasmid

vector as described further herein. Use of the term "having the binding specificity of" indicates that equivalent monoclonal antibodies exhibit the same or similar immunoreaction (binding) properties and compete for binding to a preselected target molecule.

The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies having the substituted peptide also bind to the preselected target molecule. Analogously, another preferred embodiment of the invention relates to polynucleotides which encode the above noted heavy and/or light chain peptides and to polynucleotide sequences which are complementary to these polynucleotide sequences. Complementary polynucleotide sequences include those sequences which hybridize to the polynucleotide sequences of the invention under stringent hybridization conditions.

Human monoclonal antibodies offer particular advantages over murine monoclonal antibodies, particularly insofar as they can be used therapeutically in humans. Specifically, human antibodies are not cleared from the circulation as rapidly as "foreign" antigens, and do not activate the immune system in the same manner as foreign antigens and foreign antibodies.

The invention contemplates, in one embodiment, a monoclonal antibody of this invention produced by the present methods.

In another preferred embodiment, the invention contemplates a truncated immunoglobulin molecule comprising a Fab fragment derived from a human monoclonal antibody of this invention. The Fab fragment, lacking Fc receptor, is soluble, and affords therapeutic advantages in serum half-life, and diagnostic advantages in modes of using the soluble Fab fragment. The preparation of a soluble Fab fragment is generally known in the immunological arts and can be accomplished by a variety of methods. A preferred method of producing a soluble Fab fragment is described herein.

Preferably, an antibody of this invention specifically immunoreacts with integrin

$\alpha_{IIb}\beta_3$. That is, an antibody shows preferential binding to integrin $\alpha_{IIb}\beta_3$ as compared to other integrins. Exemplary and preferred antibodies are disclosed herein and designated as RAD3, RAD4, RAD3, RAD4, RAD9, RAD11, RAD12, RAD32, RAD34, RAD87, or RAD88. The amino acid residue sequences of the VH region of those antibodies is set forth in FIG. 6.

Particularly preferred human monoclonal antibodies are those having the immunoreaction (binding) specificity of a monoclonal antibody having heavy and light chain immunoglobulin variable region amino acid residue sequences in pairs (H:L) where the light chain is the light chain described herein and the heavy chain has one of the recited sequences of Fig. 6 and conservative substitutions thereof.

In another aspect, the invention provides a peptide that inhibits platelet aggregation. The peptide has from 9 to about 50 amino acid residues and is derived from an antibody of this invention. Preferably, the peptide includes an amino acid residue sequence that corresponds to the formula V(V/W)CRAD(K/R)RC (SEQ ID NO:4). Exemplary and preferred peptides are set forth below in the examples.

The present invention contemplates therapeutic compositions useful for practicing the therapeutic methods described herein. Therapeutic compositions of the present invention contain a physiologically tolerable carrier together with at least one species of human monoclonal antibody or peptide derived therefrom as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a human patient for therapeutic purposes.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a human without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art. Typically such compositions are prepared as sterile injectables either as liquid solutions or suspensions, aqueous or non-aqueous, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified. Thus, an antibody molecule-containing composition can take the form of solutions, suspensions, tablets, capsules, sustained release formulations or powders, or other compositional forms.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the peptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous

carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, propylene glycol, polyethylene glycol, and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, organic esters such as ethyl oleate, and water-oil emulsions.

A therapeutic composition contains a human monoclonal antibody of the present invention, typically in an amount of at least 0.1 weight percent of antibody per weight of total therapeutic composition. A weight percent is a ratio by weight of antibody to total composition. Thus, for example, 0.1 weight percent is 0.1 grams of antibody per 100 grams of total composition.

Preferably, an antibody-containing therapeutic composition typically contains about 10 microgram (μg) per milliliter (ml) to about 100 milligrams (mg) per ml of antibody as active ingredient per volume of composition, and more preferably contains about 1 mg/ml to about 10 mg/ml (i.e., about 0.1 to 1 weight percent).

A therapeutic composition in another embodiment contains a peptide of the present invention, typically in an amount of at least 0.1 weight percent of peptide per weight of total therapeutic composition. A weight percent is a ratio by weight of peptide to total composition. Thus, for example, 0.1 weight percent is 0.1 grams of peptide per 100 grams of total composition.

Preferably, a peptide-containing therapeutic composition typically contains about 10 microgram (μg) per milliliter (ml) to about 100 milligrams (mg) per ml of peptide as active ingredient per volume of composition, and more preferably contains about 1 mg/ml to about 10 mg/ml (i.e., about 0.1 to 1 weight percent).

In another aspect, the present invention contemplates therapeutic methods using the compositions and compounds of the invention. In view of the benefit of using human monoclonal antibodies *in vivo* in human patients, the presently described antibodies are

particularly well suited for *in vivo* use as a therapeutic reagent for blocking or inhibiting the function of the target molecule to which the antibody binds. The peptides derived from the monoclonal antibodies described herein are also contemplated for use in the therapeutic methods of this invention. The method comprises contacting a sample believed to contain the target molecule with a composition comprising a therapeutically effective amount of a human monoclonal antibody or peptide of this invention that binds the target molecule.

For *in vivo* modalities, the method comprises administering to the patient a therapeutically effective amount of a physiologically tolerable composition containing a human monoclonal antibody or peptide of the invention.

The dosage ranges for the administration of the monoclonal antibodies and peptides of the invention are those large enough to produce the desired effect in which the disease symptoms mediated by the target molecule are ameliorated. The dosage should not be so large as to cause adverse side effects, such as hyperviscosity syndromes, pulmonary edema, congestive heart failure, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any complication.

A therapeutically effective amount of an antibody of this invention is typically an amount of antibody such that when administered in a physiologically tolerable composition is sufficient to achieve a plasma concentration of from about 0.1 microgram (μg) per milliliter (ml) to about 100 μg/ml, preferably from about 1 μg/ml to about 5 μg/ml, and usually about 5 μg/ml. Stated differently, the dosage can vary from about 0.1 mg/kg to about 300 mg/kg, preferably from about 0.2 mg/kg to about 200 mg/kg, most preferably from about 0.5 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or several days.

A therapeutically effective amount of a peptide of this invention is typically an amount of peptide such that when administered in a physiologically tolerable composition is

sufficient to achieve a plasma concentration of from about 0.1 microgram (μg) per milliliter (ml) to about 100 μg/ml, preferably from about 1 μg/ml to about 10 μg/ml. Stated differently, the dosage can vary from about 0.1 mg/kg to about 300 mg/kg, and preferably from about 0.2 mg/kg to about 200 mg/kg, in one or more dose administrations daily, for one or several days.

The human monoclonal antibodies or peptides of the invention can be administered parenterally by injection or by gradual infusion over time. Although the target molecule can typically be accessed in the body by systemic administration and therefore most often treated by intravenous administration of therapeutic compositions, other tissues and delivery means are contemplated where there is a likelihood that the tissue targeted contains the target molecule. Thus, human monoclonal antibodies or peptides of the invention can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, and can be delivered by peristaltic means.

The therapeutic compositions containing a human monoclonal antibody or a peptide of this invention are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges for systemic application are disclosed herein

and depend on the route of administration. Suitable regimes for administration are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for *in vivo* therapies are contemplated.

An anti-$\alpha_{IIb}\beta_3$ human monoclonal antibody or peptide derived therefrom containing a $\alpha_{IIb}\beta_3$-binding site can be used to *in vivo* or *in vitro* modulate the function of $\alpha_{IIb}\beta_3$ on platelets. For instance, the human monoclonal antibody or peptide can be used in a pharmaceutically acceptable composition that, when administered to a human subject in an effective amount, is capable of inhibiting the aggregation of platelets, and thereby decreasing the rate of thrombus formation. Thus, *in vivo* administration of an anti- $\alpha_{IIb}\beta_3$ human monoclonal antibody that inhibits platelet aggregation can be used *in vivo* to modulate any physiological response initiated by platelet adhesion, such as coagulation and some inflammatory responses.

When this method is carried out *in vivo*, an effective amount of an antibody or peptide composition containing a physiologically tolerable diluent and antibody molecules that immunoreact with $\alpha_{IIb}\beta_3$ and that inhibit platelet aggregation is intravenously administered to a mammal, and the mammal is maintained for a sufficient time period to allow the antibody molecules to immunoreact with any $\alpha_{IIb}\beta_3$ present and form an immunoreaction product and to allow the binding site containing the peptide to bind to $\alpha_{IIb}\beta_3$and form a peptide-receptor complex such that the normal ligand can no longer bind to the receptor.

The Examples that follow illustrate preferred embodiments of the present invention and are not limiting of the specification or claims in any way.

Cell line, proteins, and peptides.

HUVEC were purchased from Biowhittaker (Walkersville, MA) and were grown in EGM-2 (Biowhittaker) supplied with the cells. Integrin $\alpha_{IIb}\beta_3$ was purchased from Enzyme Research Laboratories (South Bend, IN). Integrins $\alpha_v\beta_3$, and $\alpha_v\beta_5$ were from Chemicon (Temecula, CA). Human fibrinogen was purchased from Sigma-Aldrich (St. Louis, MO). Peptides used in this study were synthesized by Peptron (Taejon, Korea). Human fibrinogen and peptides were biotinylated using EZ-Link-PFP-biotin (Pierce, Rockford, IL) following instructions by the manufacturer. Abciximab (ReoPro) was purchased from Eli Lilly (Indianapolis, IN).

Library generation.

Total RNA was prepared from bone marrow of six healthy donors using TRI REAGENT (Molecular Research Center, Cincinnati, OH). First strand cDNA was synthesized using the SUPERSCRIPT Preamplification System with oligo(dT) priming (Life Technologies, Gaithersburg, MD). A cDNA fragment encoding part of FR3, randomized HCDR3, and FR4 of V_H fused to C_H1 were amplified by PCR using primers neo-rad-f (GTG TAT TAC TGT GCG AGA GTG GGG NNK NNK NNK CGT GCC GAC NNK NNK NNK TAC GCT ATG GAC GTC TGG GGC) (SEQ ID NO:9) and dpseq (AGA AGC GTA GTC CGG AAC GTC) (SEQ ID NO:10) and phagemid vector pComb3X-TT (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) as template. For amplification of the cDNA fragment encoding FR1 to FR3 of V_H, the prepared human bone marrow cDNA was subjected to PCR using primers DP-47N-term (GCT GCC CAA CCA GCC ATG GCC GAG GTG CAG CTG TTG GAG TCT GGG GGA GGC TTG GTA) (SEQ

ID NO:11) and DP-47FR3 (CAC TCT CGC ACA GTA ATA CAC GGC CGT GTC CTC GGC TCT) (SEQ ID NO:12). All amplifications were performed under standard PCR conditions using *Taq* polymerase (Pharmacia, Piscataway, NJ). The two cDNA fragments were assembled by overlap extension PCR using primers lead-VH (GGC CAT GGC TGG TTG GGC AGC) (SEQ ID NO:13) and dp-Ex (GAG GAG GAG GAG GAG GAG AGA AGC GTA GTC CGG AAC GTC) (SEQ ID NO:14). A previously selected DPK-26 human kappa light chain cDNA in phagemid vector pComb3X was amplified by PCR using primers ompseq (AAG ACA GCT ATC GCG ATT GCA GTG) (SEQ ID NO:15) and leadB (GGC CAT GGC TGG TTG GGC AGC) (SEQ ID NO:16). The cDNAs encoding the heavy chain fragment library and the light chain were fused by overlap extension PCR using primers ompseq and dp-Ex. The resulting Fab encoding library was digested with *Sfi* I (Roche, Indianapolis, IN), ligated into phagemid vector pComb3X, and transformed into *E. coli* strain ER2537 (New England Biolabs, Beverly, MA) as described (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

Library selection.

A total of seven rounds of panning (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) were performed. First, five rounds of panning against immobilized human integrin $\alpha_{IIb}\beta_3$ were carried out using 100 ng of protein in 50 μl of metal buffer (25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$) for coating, 0.05% Tween20 in TBS (Tris-buffered saline) for washing, and 10 mg/ml trypsin (Becton-Dickson, Franklin Lakes, NJ) in TBS for elution. Costar 3690 96-well plates (Corning, NY) were used for panning. Trypsinization was done for 30 min at 37°C. For the sixth round of panning, 25 ng of protein in metal buffer was

used for coating and 0.5% Tween20 in TBS was used for washing. For the seventh round of panning, 12.5 ng of protein was used for coating. The plate was washed five times in the first round, ten times in the second and third round, and 15 times in the remaining four rounds. The output phage pool of each round was monitored by phage ELISA using sheep anti-M13 conjugated to horseradish peroxidase (Pharmacia) as a secondary antibody. After the last round of panning, phage were produced from single clones grown on output plates and tested for binding to integrin $\alpha_{IIb}\beta_3$ by phage ELISA as described (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

Light chain shuffling and selection.

V_κ and V_λ encoding cDNAs were amplified from the prepared human bone marrow cDNA using a previously published panel of primers (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), comprising sense primers that hybridize to sequences that encode the N-terminal amino acids of the various V_κ and V_λ families and reverse primers that hybridize to sequences that encode the C-terminal amino acids of FR4 of V_κ and V_λ respectively, which are highly conserved (Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S., and Foeller, C. (1991) *Sequences of Proteins of Immunological Interest*, U.S. Department of Health and Human Services, Public Health Service, Natl. Inst. Health, Bethesda). C_κ and C_λ encoding sequences were prepared as described (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) and fused to V_κ and V_λ encoding sequences respectively, by overlap extension PCR using primers RSC-F (GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC) (SEQ ID NO:17) and lead-B (GGC CAT GGC TGG TTG GGC AGC) (SEQ ID NO:18). Two cloning

strategies were used to replace the DPK-26 human kappa light chain in the selected Fab. In one approach, the DPK-26 human kappa light chain cDNA was removed from the phagemid vector pool obtained after the last round of panning by restriction digestion with *Sac* I and *Xba* I (New England Biolabs). Then the human light chain encoding library was digested with the same restriction enzymes, ligated into the prepared phagemid vector, and transformed into *E. coli* strain ER2537. In the second approach, heavy chain fragment encoding encoding cDNA was amplified from the phagemid vector pool obtained obtained after the last round of panning using lead-VH and dpseq primers and fused with the human light chain encoding library by overlap extension PCR using primers ompseq and dp-EX. The resulting Fab encoding library was digested with *Sfi* I, ligated into phagemid vector pComb3X, and transformed into *E. coli* strain ER2537 as described previously (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). After mixing the two light chain libraries, three rounds of panning, each with 12.5 ng coated human integrin $\alpha_{IIb}\beta_3$ were performed. The plate was washed 15 times in each round. After the last round of panning, phage were produced from single clones grown on output plates and tested for binding to integrin $\alpha_{IIb}\beta_3$ by phage ELISA as described (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

ELISA.

For production of soluble Fab, published procedures were followed (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Briefly, selected colonies were allowed to grow in 5 ml of superbroth for 6 h at 37°C. Growth was continued overnight after adding IPTG (isopropyl-beta-D-thiogalactopyranoside, Sigma-

Aldrich) to a final concentration of 1 mM. For each well on a Costar 3690 96-well plate, 100 ng of integrin $\alpha_{IIb}\beta_3$ in 50 µl metal buffer was coated at 4°C overnight. The plate was blocked by adding 3% (w/v) skim milk in TBS followed by incubation for 1 h at 37°C. Subsequently, 50 µl of the culture supernatant diluted with the same volume of 3% (w/v) skim milk in TBS, 50 µl of horseradish peroxidase conjugated rat anti-HA mAb 3F10 (Roche) diluted to 1 µg/ml in 3% skim milk in TBS, and 50 µl of ABTS substrate solution (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) were added sequentially. Before adding the reagents, the plate was washed five times with 0.05% Tween20 in TBS. The plate was incubated for 1 h at 37°C in each step. To check for crossreactivity of the selected Fab, parallel assays with coated integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ were performed using the same conditions as described above.

Fibrinogen binding inhibition assay.

Wells of a Costar 3690 96-well plate were coated with 100 ng of integrin $\alpha_{IIb}\beta_3$ in 50 µl metal buffer at 4°C overnight. The plate was washed twice with water and blocked with 160 µl of 3% (w/v) skim milk in TBS for 1 h at 37°C. After the plate was briefly washed with water, 50 µl of culture supernatant containing Fab, purified Fabs RAD87 and Abciximab, or synthetic peptides mixed with 50 µl of 1.2 µM biotinylated fibrinogen in 3% (w/v) skim milk in TBS were added to each well. The final concentrations were adjusted between 1.3 X 10^{-8} M and 8.0 X 10^{-7} M of Fabs and between 8.9 X 10^{-8} M and 9.1 X 10^{-5} M of synthetic peptides. After incubation for 2 h at 37°C followed by ten times washing with water and five times washing with 0.05% Tween20 in TBS, 50 µl of 0.5 µg/ml streptavidine-HRP (Pierce) diluted in 3% (w/v) skim milk in TBS was added and incubated for 1 h at 37°C. After washing as above, 50 µl of ABTS substrate solution (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring

Harbor Laboratory Press, Cold Spring Harbor, New York) was added. This experiment was repeated three times at each concentration to get the mean value and standard deviation.

Flow cytometry.

Peripheral blood was drawn from a healthy volunteer and collected in an ACD tube (Becton Dickinson, San Jose, CA). HUVEC were treated for 5 min with 0.05% (w/v) trypsin, 0.53 mM EDTA (Life Technologies), collected by centrifugation at 500 g for 2 min, and resuspended in 1% (w/v) BSA in PBS (phosphate buffered saline). Subsequently, Fab RAD87 or Fab Abciximab was added to the cells resuspended in 40 µl of 1% (w/v) BSA in PBS or 40 µl peripheral blood to reach a final concentration of 0.2 µM. After incubation for 40 min at room temperature followed by washing twice with 1% (w/v) BSA in PBS, 10 µl of FITC conjugated anti-human IgG polyclonal antibodies (Sigma-Aldrich) diluted in 750 µl of 1% (w/v) BSA in PBS was added and incubated for 20 min at room temperature. After washing twice with 1% (w/v) BSA in PBS, flow cytometry was performed using a FACStar instrument from Becton Dickinson (San Jose, CA).

Platelet aggregation assay.

Peripheral human blood was collected as described above. Platelet-rich plasma (PRP) was obtained from the collected peripheral blood by centrifugation at 135 g for 15 min. Subsequent centrifugation at 1,500 g for 15 min yielded platelet-poor plasma (PPP). By mixing PRP and PPP, conditioned plasma of 300,000 – 350,000 platelets per 1 µl of plasma was prepared. To 435 µl of the conditioned plasma, RAD 87 mAbs, abciximab and peptides dissolved in 15 µl of PBS were added to final concentrations between 20 nM and 100 nM of mAbs and between 0.4 µM and 90 µM of peptides. The platelet aggregation assay was done as described previously (Klinkhardt, U., Kirchmaier, C. M., Westrup, D., Breddin, H. K., Mahnel, R., Graff, J., Hild, M., and Harder, S. (2000) Differential in vitro effects of the

platelet glycoprotein IIb/IIIa inhibitors abxicimab or SR121566A on platelet aggregation, fibrinogen binding and platelet secretory parameters. *Thromb Res* 97, 201-207) using a whole blood lumi-aggregometer (Chrono-log, Havertown, PA). The impedance of each sample was monitored until a stable baseline was established (< 5 mV drift per min). To induce platelet aggregation, 9 μl of an ADP solution was added to reach a final concentration of 20 μM. Increase in impedance across a pair of electrodes over time was transmitted through an interface to a personal computer for analysis (AGGRO/LINK, Chrono-log).

Affinity measurement.

The dissociation constant of Fabs RAD87 and abciximab toward integrin $\alpha_{IIb}\beta_3$ was determined with competitive ELISA as described previously (Djavadi-Ohaniance, L., Goldberg, M. E., and Firguet, B. (1996) Measuring antibody affinity in solution. In *Antibody Engineering* (McCafferty, J., Hoogenboom, H. R., and Chiswell, J., eds) pp. 77-98, IRL Press, Oxford; Yi, K., Chung, J., Kim, H., Kim, I., Jung, H., Kim, J., Choi, I., Suh, P., and Chung, H. (1999) Expression and characterization of anti-NCA-95 scFv (CEA 79 scFv) in a prokaryotic expression vector modified to contain a *Sfi* I and *Not* I site. *Hybridoma* 18, 243-249). Briefly, wells of a Costar 3690 96-well plate were coated with 250 ng of integrin $\alpha_{IIb}\beta_3$ in 50 μl of metal buffer at 4°C overnight. The plate was washed five times with water and blocked with 160 μl of 2% BSA in PBS for 2 hr at 37 °C. Purified Fab RAD87 or Fab abciximab was mixed with integrin $\alpha_{IIb}\beta_3$ to reach a final concentration of 2×10^{-10} M. The final concentrations of integrin $\alpha_{IIb}\beta_3$ were adjusted between 1×10^{-7} M and 1×10^{-10} M. These mixtures were incubated overnight to allow equilibration. After the plate was briefly washed with water, the antibody-antigen mixtures were added to the wells and incubated for 2 h at room temperature. The plate was washed three times with 0.05% Tween20 in PBS before adding 200 ng/ml horseradish peroxidase conjugated anti-human IgG antibody (Pierce). After incubation at room temperature for 1 h, the plate was washed five times with

0.05% Tween20 in PBS before adding 50 μl of ABTS substrate solution (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). After incubation at 37°C for 2 h, the reaction was stopped by adding 50 μl of 3 N HCl. The absorbance of each well was measured at 405 nm in an ELISA plate reader. A Scatchard plot was drawn with υ/[Ag] on the y axis and υ on the x axis. [Ag] is the concentration of the free antigen; υ is the fraction of bound antibody, which was obtained by dividing the absorbance in the presence of a defined concentration of soluble antigen by the absorbance in the absence of soluble antigen. The slope of a straight line in the Scatchard plot is equal to $1/K_d$.

Generation and selection of a hybrid naïve/synthetic human Fab library.

The goal of this study was to generate synthetic human antibodies that discriminate integrin $\alpha_{IIb}\beta_3$ from other RGD-binding integrins, especially integrin $\alpha_v\beta_3$, by grafting an RAD motif flanked on both sites by three randomized amino acid residues into HCDR3. First, we used an RAD motif rather than an RGD motif as central recognition sequence. Second, a CX_9C disulfide bridge surrounding the central recognition sequence in both previous studies was removed. Third, the synthetic HCDR3 library was grafted into a naïve human VH library amplified from human bone marrow cDNA. Thus, in addition to its randomization in HCDR3, a second level of library complexity was introduced by diversifying VH.

A human antibody library with the randomized HCDR3 sequence VGXXXRADXXXYAMDV (SEQ ID NO:3), in which X stands for any of the 20 common natural amino acids, was generated in phagemid vector pComb3X. VH, the FR1 to FR3 encoding fragment of the heavy chain variable domain, was amplified by PCR using human variable heavy chain gene DP-47 specific primers and human bone marrow cDNA from six healthy donors as template. This hybrid naïve/synthetic heavy chain fragment library was

initially paired with a previously selected DPK-26 human kappa light chain. The resulting Fab library was cloned into phagemid vector pComb3X, yielding a complexity of 1 x 10^9 independent transformants. Randomly chosen clones from the unselected library confirmed the intended variety in VH and HCDR3 sequences. After seven rounds of panning on immobilized human integrin $\alpha_{IIb}\beta_3$, over 80% of the selected clones bound to integrin $\alpha_{IIb}\beta_3$ as analyzed by phage ELISA (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

The selected heavy chain fragment encoding sequences were subjected to a second selection step based on light chain shuffling. For this, the DPK-26 human kappa light chain was replaced by a human kappa and lambda light chain library amplified from the human bone marrow cDNA. The resulting naïve human light chain library was again cloned into phagemid vector pComb3X, yielding a complexity of 7 x 10^8 independent transformants. After three rounds of panning on immobilized human integrin $\alpha_{IIb}\beta_3$, all selected clones bound to integrin $\alpha_{IIb}\beta_3$ as analyzed by phage ELISA.

Ten individual clones that revealed the strongest binding were subsequently analyzed by DNA sequencing. All but one clone contained a disulfide bridge constrained loop in HCDR3 with the consensus sequence V(V/W)CRAD(K/R)RC (SEQ ID NO:4) (Table 2). The exception, clone RAD1, had the corresponding sequence THSRADRRE (SEQ ID NO:19) (Table 2). All clones revealed a DP-47 VH heavy chain fragment. Whereas four clones had the original DPK-26 human kappa light chain, six clones revealed human kappa or lambda light chains derived from the naïve human light chain library.

Biochemical and functional characterization of selected human Fabs.

Fabs expressed from the ten selected clones were tested for their reactivity with RGD-binding integrins by ELISA. All Fabs strongly bound to human integrin $\alpha_{IIb}\beta_3$ but not

to human integrins $\alpha_v\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_5$ (Fig. 1). To check their reactivity toward native integrin $\alpha_{IIb}\beta_3$ expressed on the surface of human platelets surface, all Fabs were subsequently analyzed by flow cytometry. All selected Fabs were found to bind to human platelets while irrelevant Fabs did not. As the interaction between integrin $\alpha_{IIb}\beta_3$ and fibrinogen is in part mediated by the RGD motif, we tested next whether the selected Fabs could inhibit this protein-protein interaction. For this purpose, we established a competitive ELISA assay based on immobilized integrin $\alpha_{IIb}\beta_3$, biotinylated fibrinogen, and avidin-HRP. The selected Fabs were mixed with the biotinylated fibrinogen at different concentrations and subsequently incubated with the immobilized integrin $\alpha_{IIb}\beta_3$. Avidin-HRP was used to detect biotinylated fibrinogen bound to immobilized integrin $\alpha_{IIb}\beta_3$. All selected Fabs revealed a potent inhibition of the interaction of integrin $\alpha_{IIb}\beta_3$ and fibrinogen.

We subsequently focused on Fab RAD87, which showed the strongest binding to integrin $\alpha_{IIb}\beta_3$ (Fig. 1). Fab RAD87 was expressed in *E. coli* and purified as described previously (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). As revealed by flow cytometry, Fab RAD87 bound only to human platelets but not to HUVEC cells, which mainly express integrin $\alpha_v\beta_3$ (Fig. 2). By contrast, Fab abciximab (ReoPro, Eli Lilly, Indianapolis, IN) bound to both platelets and HUVEC cells (Fig. 2), confirming its documented cross-reactivity with the two β_3 integrins (Bougie, D. W., Wilker, P. R., Wuitschick, E. D., Curtis, B. R., Malik, M., Levine, S., Lind, R. N., Pereira, J., and Aster, R. H. (2002) Acute thrombocytopenia after treatment with tirofiban or eptifibatide is associated with antibodies specific for ligand-occupied GPIIb/IIIa. *Blood* 100, 2071-2076). Fab RAD87 blocked the interaction between integrin $\alpha_{IIb}\beta_3$ and fibrinogen in a dose-dependent manner with an IC_{50} of 8.0×10^{-8} M. In a parallel experiment, Fab abciximab revealed an IC_{50} of 9.0×10^{-8} M (Fig. 3A, Table 3). Based on competitive ELISA (Suzuki, K., Sato, K., Kamohara, M., Kaku, S., Kawasaki, T., Yano, S., and Iizumi, Y. (2002)

Comparative studies of a humanized anti-glycoprotein IIb/IIIa monoclonal antibody, YM337, and abciximab on in vitro antiplatelet effect and binding properties. *Biol Pharm Bull* 25, 1006-1012; Co, M. S., Yano, S., Hsu, R. K., Landolfi, N. F., Vasquez, M., Cole, M., Tso, J. T., Bringman, T., Laird, W., Hudson, D., and et al. (1994) A humanized antibody specific for the platelet integrin gpIIb/IIIa. *J Immunol* 152, 2968-2976), the K_d value of the monovalent Fab RAD87 / integrin $\alpha_{IIb}\beta_3$ interaction was 3.3 x 10^{-9} M (Table 3). The same assay yielded a K_d value of 1.1 x 10^{-9} M for the monovalent Fab abciximab / integrin $\alpha_{IIb}\beta_3$ interaction, as compared to published 6.2 x 10^{-9} M (Table 3) (Tam, S. H., Sassoli, P. M., Jordan, R. E., and Nakada, M. T. (1998) Abciximab (ReoPro, chimeric 7E3 Fab) demonstrates equivalent affinity and functional blockade of glycoprotein IIb/IIIa and alpha(v)beta3 integrins. *Circulation* 98, 1085-1091).

As the interaction between integrin $\alpha_{IIb}\beta_3$ and fibrinogen is an essential step for platelet aggregation, we tested next whether Fab RAD87 could inhibit platelet aggregation *ex vivo*. Platelet aggregation was induced and monitored by a platelet aggregometer by adding ADP to a final concentration of 20 μM to conditioned plasma prepared from human peripheral blood. Fab RAD87 was found to potently inhibit platelet aggregation with an EC_{50} of 60 nM or 3 μg/ml (Fig. 4; Table 3). In a parallel experiment, the EC_{50} of Fab abciximab was determined to be 45 nM, which was previously reported to be 34 nM (Klinkhardt, U., Kirchmaier, C. M., Westrup, D., Breddin, H. K., Mahnel, R., Graff, J., Hild, M., and Harder, S. (2000) Differential in vitro effects of the platelet glycoprotein IIb/IIIa inhibitors abxicimab or SR121566A on platelet aggregation, fibrinogen binding and platelet secretory parameters. *Thromb Res* 97, 201-207) when platelet aggregation was induced at a concentration of 5 μM ADP.

Biochemical and functional characterization of synthetic peptides derived from the selected HCDR3 sequences.

Four nonapeptides whose sequences were derived from the selected HCDR3 sequences of the integrin $\alpha_{IIb}\beta_3$ binding Fab were chemically synthesized. This panel included three cyclic peptides, VWCRADKRC (SEQ ID NO:6), VWCRADRRC (SEQ ID NO:5), and VVCRADRRC (SEQ ID NO:7), and linear peptide THSRADRRE (SEQ ID NO:19). Using the competitive ELISA assay described above, all cyclic peptides were found to inhibit the interaction between integrin $\alpha_{IIb}\beta_3$ and fibrinogen in micromolar concentration range. The linear peptide as well as three control peptides - two with an inversed RAD motif, VVCDARRRC (SEQ ID NO:20) and THSDARRRE (SEQ ID NO:21), and one with an unrelated sequence - did not inhibit the protein-protein interaction in the same concentration range (Fig. 3B). The IC_{50} of the most potent cyclic peptides, VWCRADRRC (SEQ ID NO:5) and VWCRADKRC (SEQ ID NO:6) were determined to be 1.2×10^{-6} M and 4.2×10^{-6} M respectively. The IC_{50} of the cyclic peptide VVCRADRRC whose sequence was derived from Fab RAD87 was 1.1×10^{-5} M, which is two orders of magnitude higher than the corresponding IC_{50} obtained for Fab RAD87 (Fig. 3B).

Figure 5 shows the *ex vivo* platelet aggregation assay in the presence of various concentrations of VWCRADRRC (SEQ ID NO:5) (A), VVCRADRRC (SEQ ID NO:7) (B), VWCRADKRC (SEQ ID NO:6) (C) and THSDARRRE (SEQ ID NO:19) and the control peptides (D). Strikingly, all three cyclic peptides but neither linear nor control peptides completely inhibited platelet aggregation at a concentration of 90 μM. In correlation with the integrin $\alpha_{IIb}\beta_3$ / fibrinogen interaction assay, cyclic peptide VWCRADRRC (SEQ ID NO:5), the only peptide to inhibit platelet aggregation at a concentration as low as 9 μM (Fig. 5A), was again found to be the most potent inhibitor.

While their potential application as anti-thrombotic drugs overlaps with abciximab, the RAD antibodies we describe here have several distinctive features. First, they are

human antibodies, which are less likely to induce an immune response in the patients. Second, like RGD peptides and peptidomimetics (Xiong, J. P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S. L., and Arnaout, M. A. (2002) Crystal structure of the extracellular segment of integrin alpha Vbeta3 in complex with an Arg-Gly-Asp ligand. *Science* 296, 151-155), the RAD antibodies directly block the RGD binding site of integrin $\alpha_{IIb}\beta_3$. By contrast, the mechanism of integrin $\alpha_{IIb}\beta_3$ ligation by abciximab, which does not contain an RGD or RGD-like motif, is thought to involve steric or allosteric hindrance rather than direct blocking of the RGD binding site. Third, the RAD antibodies selectively bind to integrin $\alpha_{IIb}\beta_3$, whereas abciximab does not differentiate between integrins $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$. The cross-reactivity of abciximab is analogous to our earlier integrin binding synthetic antibodies (Barbas, C. F., 3rd, Languino, L. R., and Smith, J. W. (1993) High-affinity self-reactive human antibodies by design and selection: targeting the integrin ligand binding site. *Proc Natl Acad Sci U S A* 90, 10003-10007; Smith, J. W., Hu, D., Satterthwait, A., Pinz-Sweeney, S., and Barbas, C. F., 3rd (1994) Building synthetic antibodies as adhesive ligands for integrins. *J Biol Chem* 269, 32788-32795).

Acute thrombocytopenia whose cause is not known is one of the principal safety issues with integrin $\alpha_{IIb}\beta_3$ inhibitors. As mentioned above, human antibodies against the mouse variable domains of abciximab were reported as the main cause of platelet destruction in patients who developed severe thrombocytopenia after being given abciximab a second time (Curtis, B. R., Swyers, J., Divgi, A., McFarland, J. G., and Aster, R. H. (2002) Thrombocytopenia after second exposure to abciximab is caused by antibodies that recognize abciximab-coated platelets. *Blood* 99, 2054-2059). This finding might necessitate a further humanization of abciximab by grafting its CDRs onto the framework of human variable domains (Rader, C., Ritter, G., Nathan, S., Elia, M., Gout, I., Jungbluth, A. A., Cohen, L. S., Welt, S., Old, L. J., and Barbas, C. F., 3rd (2000) The rabbit antibody repertoire as a novel source for the generation of therapeutic human antibodies. *J Biol Chem* 275, 13668-13676).

However, if not accompanied by framework fine tuning based on detailed structural information of the antibody, this CDR grafting strategy often yields antibodies with greatly reduced affinity to the antigen as in the case of anti-integrin $\alpha_{IIb}\beta_3$ monoclonal antibody YM337, which is currently evaluated in clinical trials (Suzuki, K., Sato, K., Kamohara, M., Kaku, S., Kawasaki, T., Yano, S., and Iizumi, Y. (2002) Comparative studies of a humanized anti-glycoprotein IIb/IIIa monoclonal antibody, YM337, and abciximab on in vitro antiplatelet effect and binding properties. *Biol Pharm Bull* 25, 1006-1012; Co, M. S., Yano, S., Hsu, R. K., Landolfi, N. F., Vasquez, M., Cole, M., Tso, J. T., Bringman, T., Laird, W., Hudson, D., and et al. (1994) A humanized antibody specific for the platelet integrin gpIIb/IIIa. *J Immunol* 152, 2968-2976). As the RAD antibodies are entirely composed of human sequences, except for the synthetic HCDR3, they are expected to be less immunogenic than chimeric or humanized antibodies. However, the induction of human anti-idiotypic antibodies by our RAD antibodies is possible. Since circulating anti-idiotypic antibodies would compete with platelet integrin $\alpha_{IIb}\beta_3$ for RAD antibody binding rather than bind to the platelet surface through the RAD antibodies, it is a reasonable assumption that they would not cause severe thrombocytopenia. An interesting recent finding was that human antibodies that selectively recognized integrin $\alpha_{IIb}\beta_3$ when complexed with tirofiban and eptifibatide were found in the very limited number of patients who developed severe thrombocytopenia after being treated with the small molecule drugs (Bougie, D. W., Wilker, P. R., Wuitschick, E. D., Curtis, B. R., Malik, M., Levine, S., Lind, R. N., Pereira, J., and Aster, R. H. (2002) Acute thrombocytopenia after treatment with tirofiban or eptifibatide is associated with antibodies specific for ligand-occupied GPIIb/IIIa. *Blood* 100, 2071-2076). In this context, the possibility remains that RAD antibodies could cause thrombocytopenia by inducing the display of immunogenic epitopes on integrin $\alpha_{IIb}\beta_3$ as their binding mode mimics the small molecule drugs.

Several mouse mAbs specific for integrin $\alpha_{IIb}\beta_3$, including LJ-CP3, OPG2, and PAC-1 (Puzon-McLaughlin, W., Kamata, T., and Takada, Y. (2000) Multiple discontinuous ligand-mimetic antibody binding sites define a ligand binding pocket in integrin alpha(IIb)beta(3). *J Biol Chem* 275, 7795-7802; Kamata, T., Irie, A., Tokuhira, M., and Takada, Y. (1996) Critical residues of integrin alphaIIb subunit for binding of alphaIIbbeta3 (glycoprotein IIb-IIIa) to fibrinogen and ligand-mimetic antibodies (PAC-1, OP-G2, and LJ-CP3). *J Biol Chem* 271, 18610-18615; Prammer, K. V., Boyer, J., Ugen, K., Shattil, S. J., and Kieber-Emmons, T. (1994) Bioactive Arg-Gly-Asp conformations in anti-integrin GPIIb-IIIa antibodies. *Receptor* 4, 93-108; Tomiyama, Y., Brojer, E., Ruggeri, Z. M., Shattil, S. J., Smiltneck, J., Gorski, J., Kumar, A., Kieber-Emmons, T., and Kunicki, T. J. (1992) A molecular model of RGD ligands. Antibody D gene segments that direct specificity for the integrin alpha IIb beta 3. *J Biol Chem* 267, 18085-18092; Niiya, K., Hodson, E., Bader, R., Byers-Ward, V., Koziol, J. A., Plow, E. F., and Ruggeri, Z. M. (1987) Increased surface expression of the membrane glycoprotein IIb/IIIa complex induced by platelet activation. Relationship to the binding of fibrinogen and platelet aggregation. *Blood* 70, 475-483; Bennett, J. S., Hoxie, J. A., Leitman, S. F., Vilaire, G., and Cines, D. B. (1983) Inhibition of fibrinogen binding to stimulated human platelets by a monoclonal antibody. *Proc Natl Acad Sci U S A* 80, 2417-2421) contain an RYD motif in HCDR3. The binding of these mouse mAbs to integrin $\alpha_{IIb}\beta_3$ could be completely blocked by RGD peptides, suggesting that their RYD motif mediates a direct interaction with the RGD binding site. Interestingly, mouse mAb, 16N7C2, which contains an RGD motif in HCDR3, did not differentiate between the β_3 integrins (Deckmyn, H., Stanssens, P., Hoet, B., Declerck, P. J., Lauwereys, M., Gansemans, Y., Tornai, I., and Vermylen, J. (1994) An echistatin-like Arg-Gly-Asp (RGD)-containing sequence in the heavy chain CDR3 of a murine monoclonal antibody that inhibits human platelet glycoprotein IIb/IIIa function. *Br J Haematol* 87, 562-571). Thus, similar to our synthetic RAD motif, the native RYD motif in some contexts provide for selective recognition of integrin $\alpha_{IIb}\beta_3$.

However, in contrast to the synthetic RAD antibodies, neither LJ-CP3, OPG2, or PAC-1 contain a disulfide bridge in HCDR3 that displays the RGD-like motif (Tomiyama, Y., Brojer, E., Ruggeri, Z. M., Shattil, S. J., Smiltneck, J., Gorski, J., Kumar, A., Kieber-Emmons, T., and Kunicki, T. J. (1992) A molecular model of RGD ligands. Antibody D gene segments that direct specificity for the integrin alpha IIb beta 3. *J Biol Chem* 267, 18085-18092) suggesting either a limitation in the structural diversity that can be achieved by VDJ recombination or a selection against disulfide bridges in HCDR3. Previously, we selected human antibodies with a synthetically grafted RGD motif or RGD-mimicking motif in HCDR3 for binding to integrins $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ (Barbas, C. F., 3rd, Languino, L. R., and Smith, J. W. (1993) High-affinity self-reactive human antibodies by design and selection: targeting the integrin ligand binding site. *Proc Natl Acad Sci U S A* 90, 10003-10007; Smith, J. W., Hu, D., Satterthwait, A., Pinz-Sweeney, S., and Barbas, C. F., 3rd (1994) Building synthetic antibodies as adhesive ligands for integrins. *J Biol Chem* 269, 32788-32795; Barbas, C. F., 3rd (1993) Recent advances in phage display. *Curr Opin Biotechnol* 4, 526-530). None of the selected antibodies were exclusively specific for either integrin. In an attempt to select antibodies with higher specificity, we generated a new synthetic human antibody library with the randomized HCDR3 sequence VGXXXRADXXXYAMDV (SEQ ID NO:3). In addition to replacing the RGD-motif by an RAD motif, an important distinction of the new library was the removal of a CX_9C disulfide bridge that embraced the integrin binding motif and its flanking residues in previous libraries. Interestingly, the selection of the new library against integrin $\alpha_{IIb}\beta_3$ yielded a new motif-displaying disulfide bridge of the type CX_5C that was found in 90% of the selected antibody sequences. This smaller loop structure, whose selection within the previous CX_9C disulfide bridge would have been very unlikely, if not impossible, resulted in an exceptional selectivity toward integrin $\alpha_{IIb}\beta_3$.

The structural constraints of the selected XXXRADXXX (SEQ ID NO:22) motifs within HCDR3 prompted us to dissect them from the antibody scaffold and evaluate their

functional properties. Three synthetic peptides that display the RAD motif within a CX_5C disulfide bridge, VWCRADRRC (SEQ ID NO:5), VWCRADKRC (SEQ ID NO:6), and VVCRADRRC (SEQ ID NO:7), but not the linear synthetic peptide THSRADRRE (SEQ ID NO:19), inhibited the binding of the selected RAD antibodies to integrin $\alpha_{IIb}\beta_3$, the binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$, as well as platelet aggregation. Peptide antagonists for integrin $\alpha_{IIb}\beta_3$ have been selected from peptide libraries by phage display (O'Neil, K. T., Hoess, R. H., Jackson, S. A., Ramachandran, N. S., Mousa, S. A., and DeGrado, W. F. (1992) Identification of novel peptide antagonists for GPIIb/IIIa from a conformationally constrained phage peptide library. *Proteins* 14, 509-515; Koivunen, E., Wang, B., and Ruoslahti, E. (1995) Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins. *Biotechnology (N Y)* 13, 265-270; Koivunen, E., Restel, B. H., Rajotte, D., Lahdenranta, J., Hagedorn, M., Arap, W., and Pasqualini, R. (1999) Integrin-binding peptides derived from phage display libraries. *Methods Mol Biol* 129, 3-17). Constrained peptide libraries of the type CX_5C, CX_6C, CX_7C, and CX_9 were used. However, only the CX_6C and CX_7C peptide libraries yielded binders to integrin $\alpha_{IIb}\beta_3$. The selected sequences could be categorized into two groups, those containing an RGD motif and those containing an RGD-like motif, in which either the glycine or arginine residue of RGD was replaced. The central glycine was substituted by a variety of different amino acid residues such as serine, threonine, leucine, alanine, glutamine, histidine, and methionine. Two peptides; CRADVPLC (SEQ ID NO:23) and CMSRADRPC (SEQ ID NO:24) contained an RAD motif. The RGD-containing sequences selected on integrin $\alpha_{IIb}\beta_3$ differed from those selected on integrin $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in that aromatic residues Trp, Phe, or Tyr were enriched at the position immediately C-terminal to the RGD motif. In addition, several sequences contained one or two basic residues outside the RGD motif. However, none of the selected peptide sequences shared similarity with our selected HCDR3 sequences VWCRADKRC (SEQ ID NO:6), VWCRADRRC (SEQ ID NO:5), and VVCRADRRC (SEQ ID NO:7). Of

note is the fact that the CX_5C peptide library, which includes our CX_5C core consensus sequence CRAD(K/R)RC, did not yield any binding peptide to integrin $\alpha_{IIb}\beta_3$, suggesting that the N-terminal VW or VV residues in our selected HCDR3 sequences play an essential role in the interaction with integrin $\alpha_{IIb}\beta_3$.

Phage display of both peptide and antibody libraries has become a standard technology for a variety of applications in research and development (Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Kay, B. K., Kasanov, J., and Yamabhai, M. (2001) Screening phage-displayed combinatorial peptide libraries. *Methods* 24, 240-246; Sidhu, S. S. (2000) Phage display in pharmaceutical biotechnology. *Curr Opin Biotechnol* 11, 610-616). The display of peptide libraries within the antibody immunoglobulin variable domain merges these technologies, providing an intriguing link between antibody, peptide, and peptidomimetic drug discovery. Here we demonstrated the efficacy of this approach to generate novel specific anti-receptor peptides and antibodies. The placement of bioactive and or binding peptides within an antibody scaffold or their generation within the scaffold provides for the rapid development of immunological agents that can be used as biological tools or therapeutics (Barbas, C. F., 3rd (1993) Recent advances in phage display. *Curr Opin Biotechnol* 4, 526-530). Often peptides themselves have compromised activity *in vivo* and their binding may be difficult to monitor, however, their display within the context of an antibody addresses detection problems as well as problems associated with proteolysis of peptides and their rapid clearance since antibodies and antibody fragments exhibit relatively predictable pharmacokinetic behavior. Where it is desirable, for example in a cancer setting, the Fc region of the antibody can bestow cell-killing properties onto the peptide sequence as a result of immune effector coupling. We believe that this approach can be applied to a wide range of peptides with binding activity to rapidly generate useful immunological reagents (Brown, K. C. (2000) New approaches for

cell-specific targeting: identification of cell-selective peptides from combinatorial libraries. *Curr Opin Chem Biol* 4, 16-21).

Table 1. HCDR3 sequences from synthetic human antibody libraries selected against β_3 integrins

Anti-gp120 Fab	VGP YSW DDS PDQ NYYMDV
Fab library[a]	VGC XXX RGD XXX CYYMDV
Fab-4	--- TGQ --- WRS ------
Fab-7	--- TYG --- TRN ------
Fab-8	--- PIP --- WRE ------
Fab-9	--- SFG --- IRN ------
Fab-10	--- TWG --- ERN ------
Fab-9	VGC SFG RGD IRN CYYMDV
MTF library[b]	VGC SFG XXX XRN CYYMDV
MTF-2	--- --- RTD Q-I ------
MTF-10	--- --- KGD N-I ------
MTF-32	--- --- RRD E-- ------
MTF-40	--- --- RND S-- ------
MTF-1	--- --- RVD D-- ------
MTF-12	--- --- RAD R-- ------
MTF-15	--- --- RSV D-- ------
MTF-7	--- --- KRD M-- ------
MTF-13	--- --- RWD A-- ------
MTF-14	--- --- RQD V-- ------
MTF-20	--- --- RDD G-- ------
RAD library	VR XXX RAD XXX YAMDV

[a]Barbas et al.(18); [b]Smith et al.(19)

Table 2. Sequences of RAD library Fabs selected against integrin $\alpha_{IIb}\beta_3$

Fab	VH	HCDR3	VL	
RAD1	VH3 DP-47	VRTHSRADRREYAMDV	VKIII	DPK22/A27
RAD3	VH3 DP-47	VRVVCRADRRCYAMDV	VKVI	DPK26/A26
RAD4	VH3 DP-47	VGVWCRADRRCYAMDV	VKVI	DPK26/A26
RAD9	VH3 DP-47	VRVVCRADRRCYAMDV	VKIII	Vg/38K
RAD11	VH3 DP-47	VGVWCRADRRCYAMDV	VkVI	DPK26/A26
RAD12	VH3 DP-47	VRVVCRADRRCYAMDV	VL8	8a.88E1/DPL21
RAD32	VH3 DP-47	VGVWCRADKRCYAMDV	VKIII	3A9
RAD34	VH3 DP-47	VRVVCRADRRCYAMDV	VL3	V2-14
RAD87	VH3 DP-47	VGVVCRADRRCYAMDV	VL2	2c.118D9/v1-2
RAD88	VH3 DP-47	VRVWCRADKRCYAMDV	VKVI	DPK26/A26

Table 3. Comparison of Fab RAD87 and Fab abciximab

Fab	K_d[a]	IC_{50}[b]	EC_{50}[c]
RAD87	3.3×10^{-9} M	8.0×10^{-8} M	60 nM
Abciximab	1.1×10^{-9} M	9.0×10^{-8} M	45 nM

[a]Dissociation constant for human integrin $\alpha_{IIb}\beta_3$ binding.

[b]Required concentration for 50 % inhibition in an interaction assay of human fibrinogen and human integrin $\alpha_{IIb}\beta_3$.

[c]Required concentration for 50 % inhibition in a human platelet aggregation assay.

WHAT IS CLAIMED IS:

1. An isolated and purified peptide inhibitor of platelet aggregation, the peptide comprising from 9 to about 50 amino acid residues and having an amino acid residue sequence that corresponds to V(V/W)CRAD(K/R)RC.

2. The peptide of claim 1 comprising the amino acid residue sequence of any of SEQ ID NOs:5-7.

3. The peptide of claim 2 consisting of any of SEQ ID NOs:5-7.

4. The peptide of claim 2 consisting essentially of any of SEQ ID NOs:5-7.

5. An antibody that specifically immunoreacts with aIIBb3 and comprises the amino acid residue sequence V(R/G)V(V/W)CRAD(R/K)RCYAMDV within a complementarity determining region of the antibody.

6. The antibody of claim 5 wherein the complementarity determining region is located in a heavy chain of the antibody.

7. The antibody of claim 5 wherein the complementarity determining region is HCDR3.

8. The antibody of claim 5 designated herein as RAD3, RAD4, RAD9, RAD11, RAD12, RAD32, RAD34, RAD87, or RAD88.

9. The antibody of claim 5 that is a human antibody.

10. An antibody having the immunoreactivity of the antibody of claim 8.

11. A method of inhibiting platelet aggregation comprising contacting platelets with an effective inhibitory amount of the peptide of claim 1.

12. A method of inhibiting platelet aggregation comprising contacting platelets with an effective inhibitory amount of the antibody of claim 5.

13. A method of inhibiting binding of fibrinogen to platelets comprising contacting the platelets with an effective inhibitory amount of the peptide of claim 1.

14. A method of inhibiting platelet aggregation comprising contacting platelets with an effective inhibitory amount of the antibody of claim 5.

Abstract of the Disclosure

The present invention provides integrin $\alpha_{IIb}\beta_3$ specific antibodies and peptides. The antibodies and peptides demonstrate little or no immunoreactivity with other integrins. Methods for inhibiting platelet aggregation using the antibodies and peptides are also provided.

Fig.1

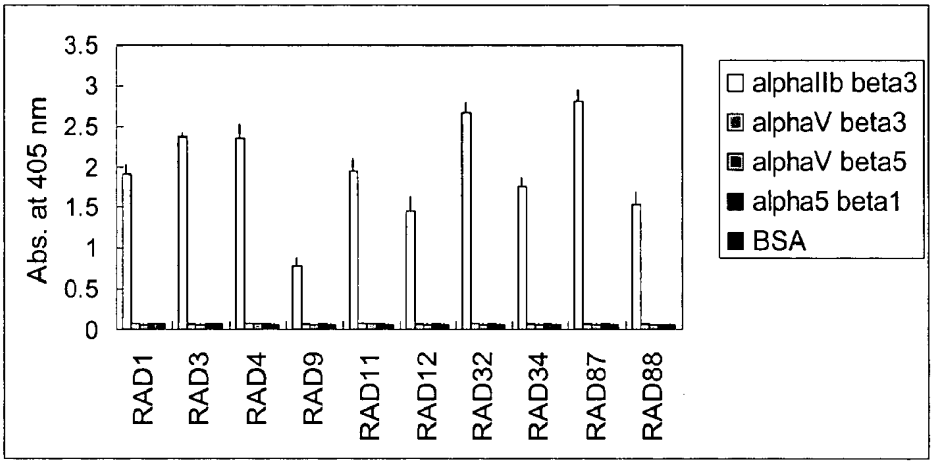

Abs. at 405 nm
3.5
3
2.5
2
1.5
1
0.5
0
RAD1
RAD3
RAD4
RAD9
RAD11
RAD12
RAD32
RAD34
RAD87
RAD88
alphaIIb beta3
alphaV beta3
alphaV beta5
alpha5 beta1
BSA

Fig. 2

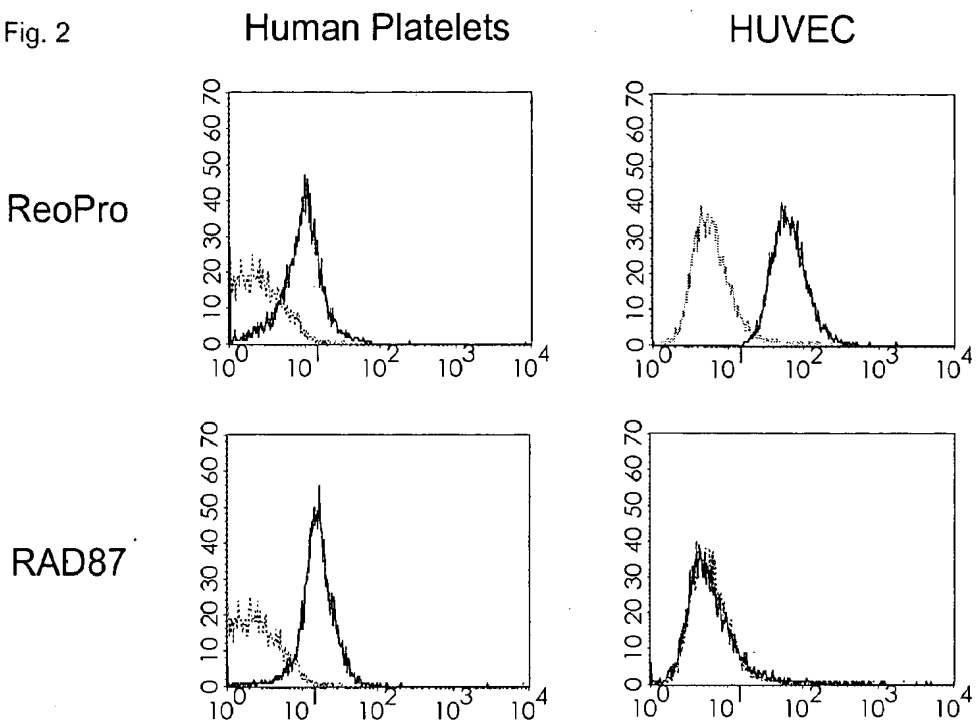
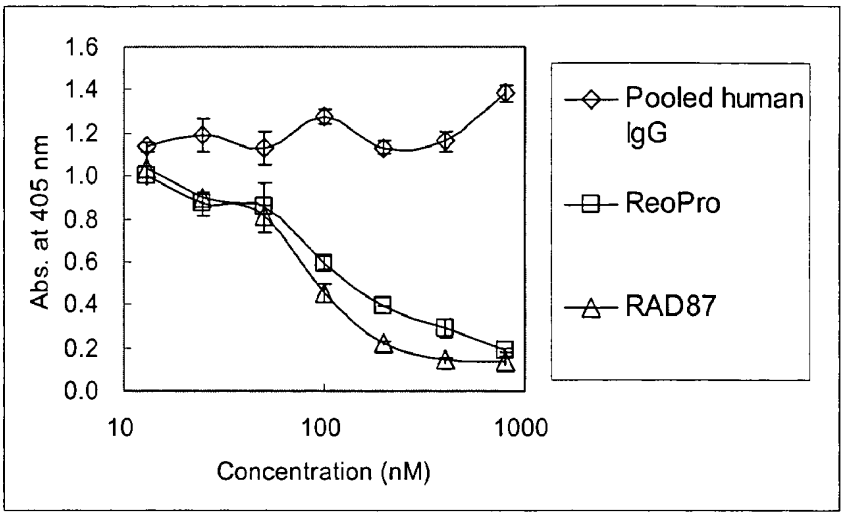

Human Platelets
HUVEC
ReoPro
RAD87
0 10 20 30 40 50 60 70
10^0 10^1 10^2 10^3 10^4

Fig.3

A


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Abs. at 405 nm
10
100
1000
Concentration (nM)
Pooled human IgG
ReoPro
RAD87

B

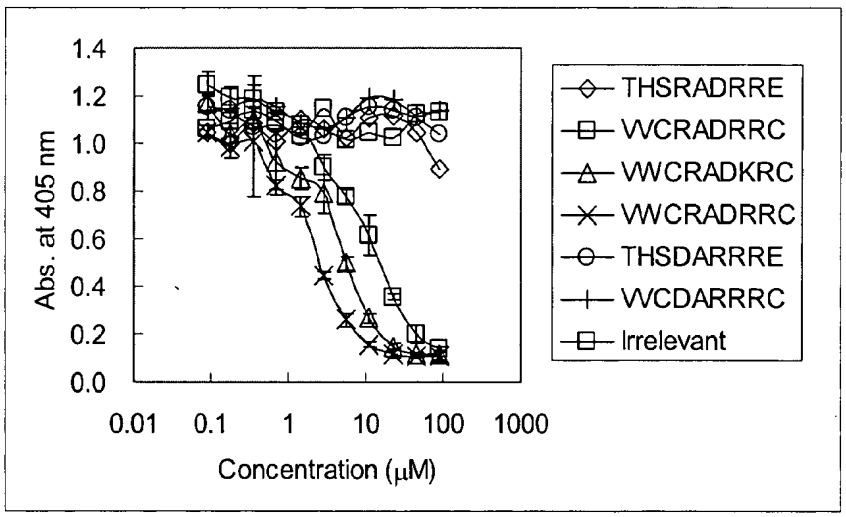
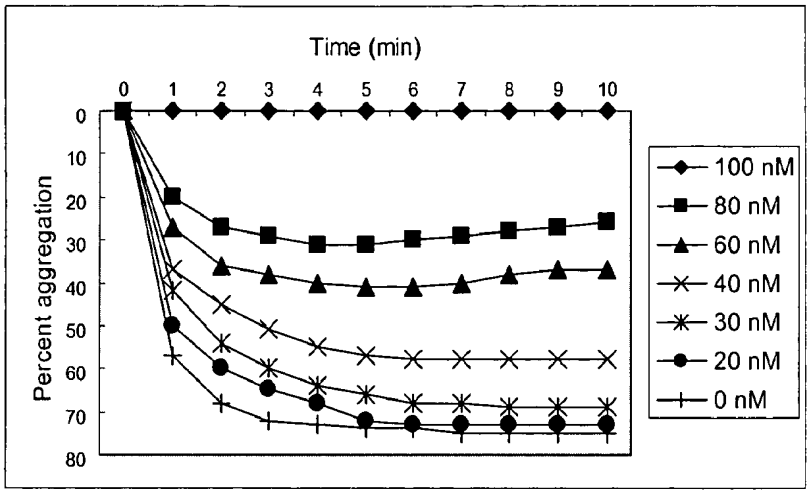
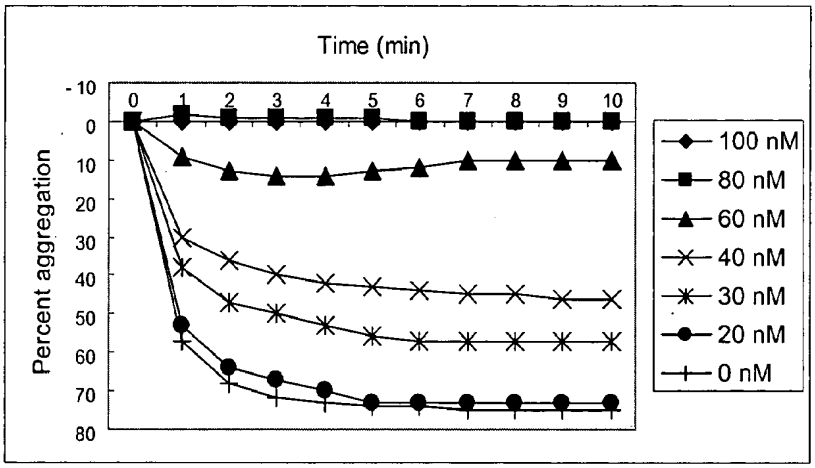

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Abs. at 405 nm
0.01
0.1
1
10
100
1000
Concentration (μM)
THSRADRRE
VVCRADRRC
VWCRADKRC
VWCRADRRC
THSDARRRE
VVCDARRRC
Irrelevant

Fig.4

A


Time (min)
0 1 2 3 4 5 6 7 8 9 10
0 10 20 30 40 50 60 70 80
Percent aggregation
100 nM
80 nM
60 nM
40 nM
30 nM
20 nM
0 nM

B


Time (min)
0 1 2 3 4 5 6 7 8 9 10
-10 0 10 20 30 40 50 60 70 80
Percent aggregation
100 nM
80 nM
60 nM
40 nM
30 nM
20 nM
0 nM

C

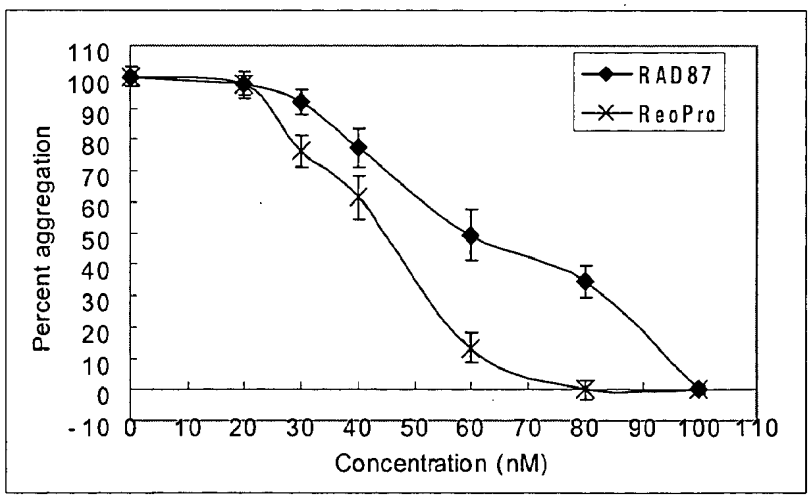

110 100 90 80 70 60 50 40 30 20 10 0 -10
0 10 20 30 40 50 60 70 80 90 100 110
Percent aggregation
Concentration (nM)
RAD87
ReoPro

Fig.5

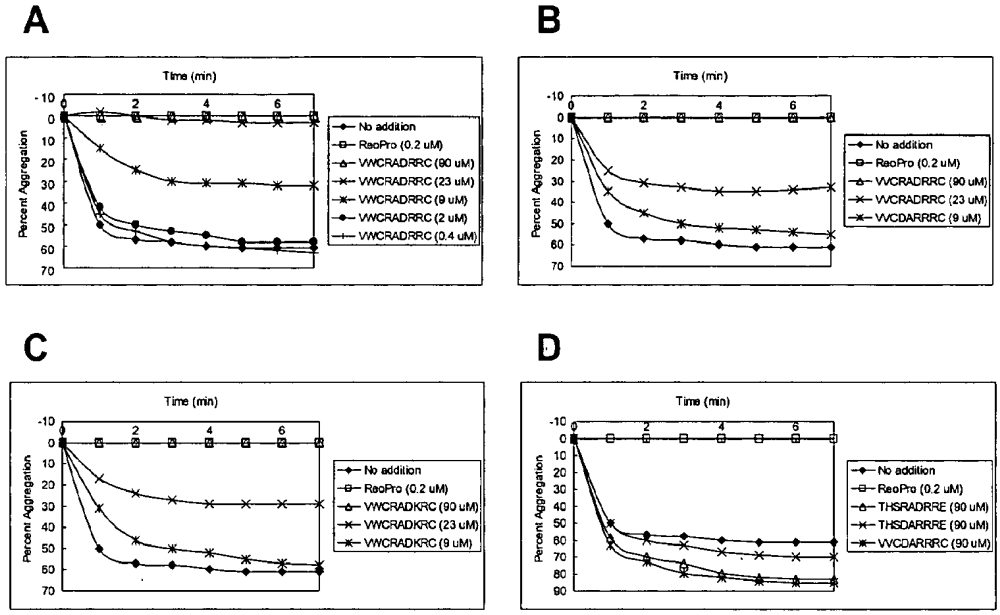
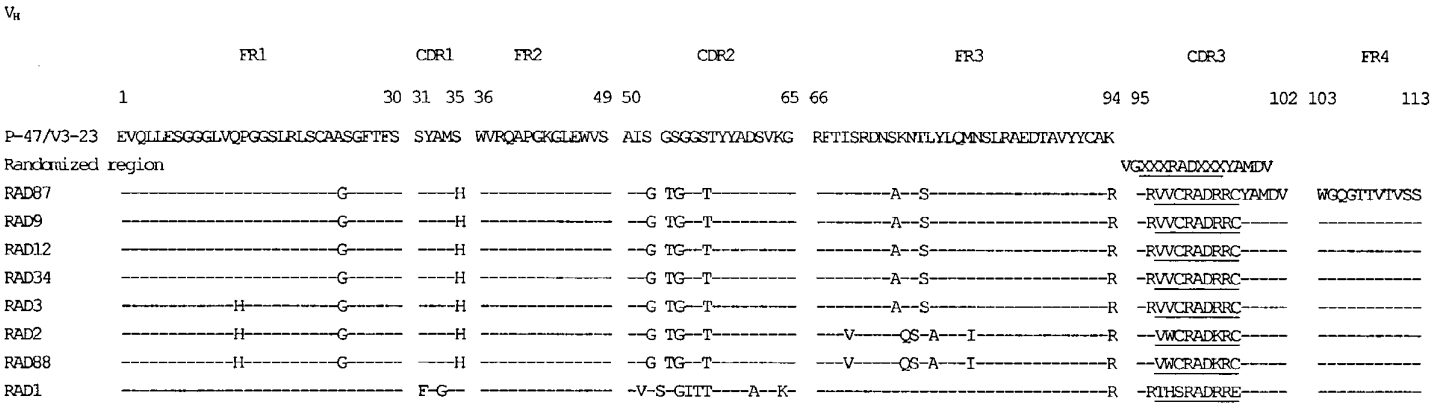

A
Time (min)
Percent Aggregation
No addition
RaoPro (0.2 uM)
VWCRADRRC (90 uM)
VWCRADRRC (23 uM)
VWCRADRRC (9 uM)
VWCRADRRC (2 uM)
VWCRADRRC (0.4 uM)
B
Time (min)
Percent Aggregation
No addition
RaoPro (0.2 uM)
VVCRADRRC (90 uM)
VVCRADRRC (23 uM)
VVCDARRRC (9 uM)
C
Time (min)
Percent Aggregation
No addition
RaoPro (0.2 uM)
VWCRADKRC (90 uM)
VWCRADKRC (23 uM)
VWCRADKRC (9 uM)
D
Time (min)
Percent Aggregation
No addition
RaoPro (0.2 uM)
THSRADRRE (90 uM)
THSDARRRE (90 uM)
VVCDARRRC (90 uM)

Fig. 6


V_H
FR1
CDR1
FR2
CDR2
FR3
CDR3
FR4
1 30 31 35 36 49 50 65 66 94 95 102 103 113
P-47/V3-23 EVQLLESGGGLVQPGGSLRLSCAASGFTFS SYAMS WVRQAPGKGLEWVS AIS GSGGSTYYADSVKG RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK
Randomized region
VGXXXRADXXXYAMDV
RAD87 -RVVCRADRRCYAMDV WGQGTTVTVSS
RAD9 -RVVCRADRRC
RAD12 -RVVCRADRRC
RAD34 -RVVCRADRRC
RAD3 -RVVCRADRRC
RAD2 --VWCRADKRC
RAD88 --VWCRADKRC
RAD1 -RTHSRADRRE